

Potential zoonotic transmission and environmental factors associated with *Blastocystis* sp. occurrence in wild roe deer (*Capreolus capreolus*) from Central Poland

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Abstract

Blastocystis is one of the most common protists colonizing the gastrointestinal tract of humans and animals worldwide. The aim of this study was to analyze the occurrence of *Blastocystis* in wild roe deer (*Capreolus capreolus*) from Central Poland, as well as to determine its zoonotic potential and the influence of selected environmental factors on the infection level. The aim of this study was to analyze the occurrence of *Blastocystis* in wild roe deer (*Capreolus capreolus*) from Central Poland, as well as to determine its zoonotic potential and the influence of selected environmental factors on the infection level. A total of 151 fecal samples were collected and analyzed using PCR amplification followed by DNA sequencing. To identify predictors of *Blastocystis* occurrence, data on animal characteristics, season, and landscape structure were compiled. Variables were analyzed using a binary generalized linear model with AIC selection. *Blastocystis* was detected in 49 samples (32.5%). Sequence analysis revealed three subtypes: ST14 (53.1%), ST13 (32.7%), and ST10 (10.2%), while two samples (4.0%) remained classified at the genus level. Roe deer present on wind farms ($p=0.016$) and in areas with a higher percentage of settlements ($p=0.035$) had a significantly lower probability of colonization. Conversely, the probability of *Blastocystis* presence increased with a higher proportion of arable land ($p=0.012$) and surface water ($p=0.024$). Although a zoonotic risk exists, its potential in the studied population appears to be low. The transmission risk may be influenced by factors related to landscape structure and anthropogenic pressure.

Keywords *Blastocystis* · Roe deer · Zoonotic potential · Environmental factors

Introduction

Blastocystis is one of the most common protists colonizing the gastrointestinal tract of humans and animals worldwide^{1,2}. The presence of *Blastocystis* in humans has been associated mainly with gastrointestinal symptoms, as well as being commonly found in asymptomatic individuals. Therefore, its pathogenicity is controversial^{2,3}. It

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has also been suggested that, in some individuals, *Blastocystis* colonization may be associated with a healthy gut microbiota^{2,4}.

Blastocystis isolates are indistinguishable morphologically; however, they show high genetic diversity. Based on polymorphism of the small subunit ribosomal RNA (18 S rRNA) gene, 49 *Blastocystis* subtypes (STs) have been reported in the literature^{5–8}. Of these, 32 subtypes (ST1–ST17, ST21, ST23–ST32, ST42–ST44, ST49) have been acknowledged as legitimate subtypes according to the criteria of having SSU rDNA sequences that differ by 4% or more^{3,9}. The vast majority of human colonization reported worldwide is caused by ST1–ST4¹⁰; nonetheless, the low host specificity demonstrated by some subtypes shared between humans and animals implies potential bidirectional transmission^{3,11,12}.

Blastocystis transmission is via the fecal-oral route, through contact with infected humans/domestic and wild animals or through ingestion of contaminated water and food^{13,14}. Colonization with the same subtypes of *Blastocystis* has been demonstrated in both zoo animals and their animal keepers, as well as in humans using the same water sources as livestock^{13,15}. The same subtypes were also found in both humans and in recreational waters contaminated with feces (from both human and/or non-human hosts)¹⁶. In Poland, most studies have focused on animals under human care, including those in zoos, farms, and veterinary clinics^{17,18}. However, wild ungulate communities, such as deer, can share habitats with domestic species and occur in close proximity to human settlements. *Blastocystis* has been identified in a wide range of wild cervids worldwide¹. Data on subtype occurrence in Polish deer remain limited¹⁹.

A clearer understanding of *Blastocystis* subtype distribution in wild populations, particularly ungulates, is needed to clarify their potential role as reservoirs for human colonization and to better understand the transmission dynamics of this protist.

The roe deer (*Capreolus capreolus*) is the most abundant cervid species in Europe²⁰. It is a small to medium-sized ungulate, with an adult body mass of 15–35 kg, a body length of 95–138 cm, and a shoulder height of 60–70 cm²¹. Roe deer exhibit considerable vagility, dispersing into suitable habitats when available, although geographic expansion can be constrained by natural barriers²¹. Over recent decades, the species has shifted beyond its conventional ecotone habitats such as forest edges to occupy diverse landscapes, including woodlands, agricultural fields, and suburban environments^{22–24}. Moreover, as an increasingly synanthropic species, it thrives in modern agroecosystems, where nutritional resources can exceed those found in contiguous forest tracts^{25–29}. According to the Statistical Yearbook of Forestry, the current roe deer population in Poland is estimated to exceed 900,000 individuals³⁰. This population grows by an average of 15% every five years, leading to localized excessive densities.

The species is increasingly expanding into human-dominated landscapes, frequently foraging in suburban areas, where it comes into close contact with humans, domestic pets, and livestock³¹. These conditions may heighten the risk of pathogen transmission from roe deer to humans and domestic animals³².

Globally, *Blastocystis* has been detected in wild Cervidae, with an overall prevalence of 24.65%³³. Across this host family, at least 22 subtypes have been identified (ST1–ST5, ST7, ST10, ST11, ST13, ST14, ST21, ST23–ST26, ST30–ST32, ST42–ST44, and ST49)^{1,33–35}. Among the cervid-associated subtypes, ST10 and ST14 carry zoonotic potential, having been detected occasionally in humans with close animal contact^{36,37}. In addition, cervids sporadically host clinically relevant human subtypes (ST1–ST5), including ST1, which may be linked to diarrhea-predominant irritable bowel syndrome (IBS-D)^{35,38}.

Blastocystis subtypes do not appear to be host specific. Various transmission routes have been investigated^{39,40}, and the presence of certain subtypes in both humans and animals suggests their zoonotic potential^{12,41}.

Data on the occurrence of *Blastocystis* in free-ranging roe deer remain limited. Therefore, the potential role of this host as a zoonotic reservoir requires further investigation.

The aim of the present work is to provide the first comprehensive study on the occurrence of *Blastocystis* in wild roe deer in Poland, as well as to determine the zoonotic potential and the influence of selected environmental factors on the infection level.

Results

Subtype distributions of *Blastocystis* in roe deer

The study population included 84 females (55.6%) and 67 males (44.4%), with a marked predominance of adults (88.7%, $n = 134$) compared to yearlings (11.3%, $n = 17$). The highest proportion of samples were collected during winter (42.4%, $n = 64$) and summer (31.1%, $n = 47$), while those obtained in autumn and spring accounted for 16.6% ($n = 25$) and 9.9% ($n = 15$) of the samples, respectively. In this study, 151 stool samples were examined, of which 49 (32.5%) tested positive for *Blastocystis*. A total of three subtypes were identified, with ST14 being the most prevalent ($n = 26$), followed by ST13 ($n = 16$) and ST10 ($n = 5$). In two cases, the subtype could not be determined because the sequences were too short, and they were therefore designated as *Blastocystis* sp. The presence of *Blastocystis* was confirmed in all of three study locations. In Location 1 (Węgrów), the dominant subtype was ST14 ($n = 12$), followed by ST13 ($n = 7$); while ST10 was not detected. In Location 2 (Rawa Mazowiecka), ST14 was also the most frequent ($n = 9$), while ST10 and ST13 were detected with the same frequency ($n = 2$ each). In Location 3 (Iłża), ST13 was the most common ($n = 7$), followed by ST14 ($n = 5$) and ST10 ($n = 3$) (Table 1).

Genetic diversity of *Blastocystis* subtypes

Phylogenetic analysis (Fig. 1), based on partial small subunit ribosomal RNA (SSU rRNA) sequences of *Blastocystis*, demonstrated that the sequences obtained in this study clustered with reference sequences within their respective subtype clades.

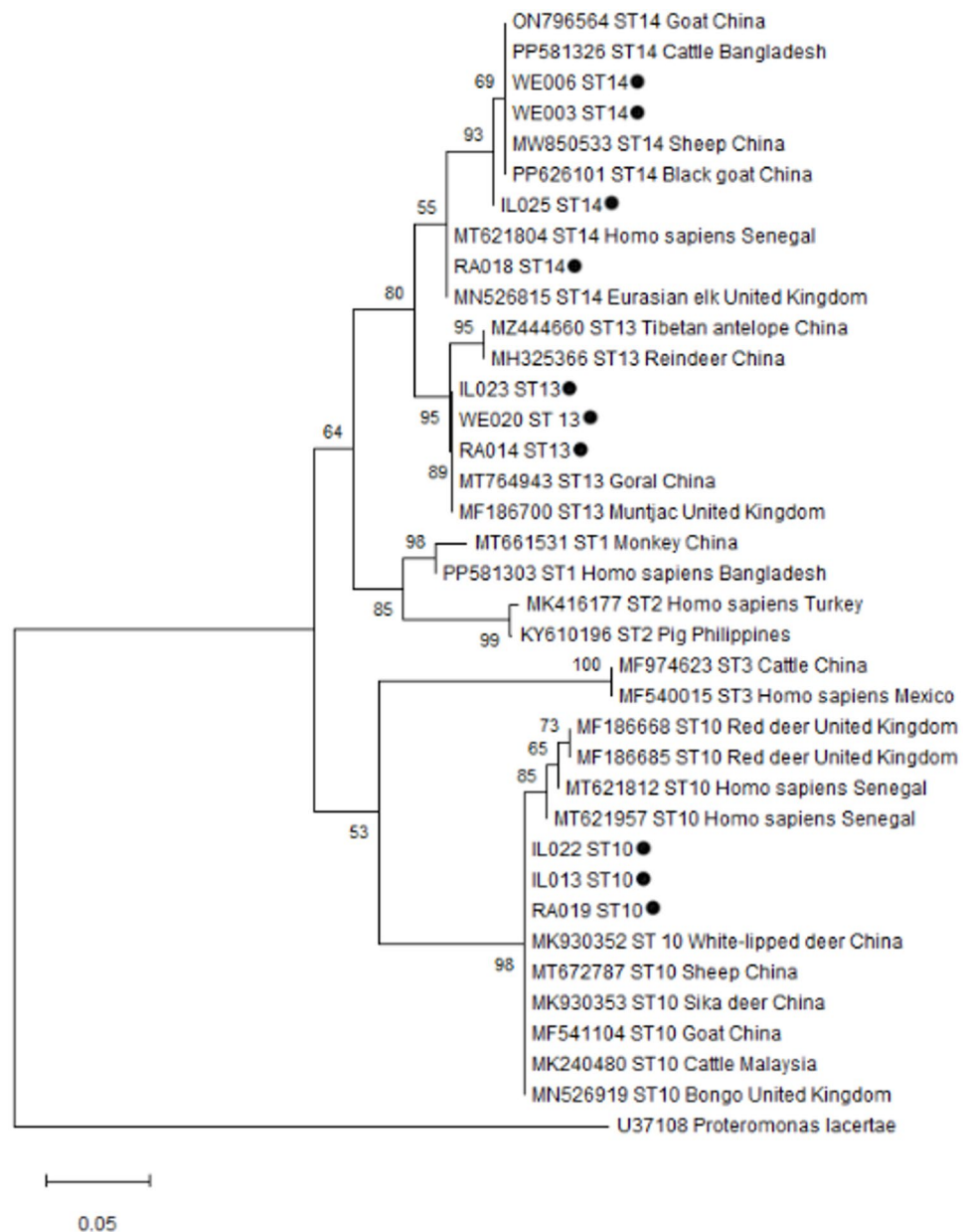
The analysis confirmed the presence of three distinct subtypes (ST10, ST13, and ST14) showing high nucleotide identity to previously published reference sequences. Four isolates (WE003, WE006, IL025, and RA018) were assigned to ST14, exhibiting 99.65–100% identity to homologues obtained from domestic and wild ruminants in China (MW850533, ON796564, PP626101), Bangladesh (PP581326), and the United Kingdom (MN526815). Three isolates (IL023, WE020, and RA014) belonged to ST13, sharing 99.14–100% identity with isolates from Chinese goral (MT764943) and the United Kingdom muntjac (MF186700). Additionally, three isolates were classified as ST10: IL013, IL022, and RA019 showing 99.81–100% identity to references from wild and domestic ruminants in China (MK930352, MK930353, MT672787, MF541104), Malaysia (MK240480), and the UK (MN526919).

These sequences generated in this study were deposited in GenBank under accession numbers PV982219–PV982267.

Table 1 Distribution of *Blastocystis* subtypes in wild roe deer (*Capreolus capreolus*) in Poland.

Factor	Category	No. (%) Examined	No. (%) Positive	ST10	ST13	ST14	ST?
Region	Locality 1 Węgrów	43 (28.5)	19 (44.2)	-	7	12	-
	Locality 2 Rawa Mazowiecka	45 (29.8)	14 (31.1)	2	2	9	1
	Locality 3 Iłża	63 (41.7)	16 (25.4)	3	7	5	1
Gender	Female	84 (55.6)	26 (31.0)	5	8	12	1
	Male	67 (44.4)	23 (34.3)	-	8	14	1
Season	Autumn	25 (16.6)	8 (32.0)	2	2	3	1
	Winter	64 (42.4)	20 (31.3)	3	6	11	-
	Summer	47 (31.1)	19 (40.4)	-	8	11	-
	Spring	15 (9.9)	2 (13.3)	-	-	1	1
Age group	Yearling	17 (11.3)	3 (17.6)	-	1	2	-
	Adult	134 (88.7)	46 (34.3)	5	15	24	2
Total		151 (100)	49 (32.5)	5	16	26	2

Fig. 1 Phylogenetic tree based on sequences of the partial small subunit ribosomal RNA (SSU rRNA) of *Blastocystis*. The phylogenetic tree was constructed using the maximum likelihood method and K2 + G substitution model. Sequence of *Proteromonas lacertae* served as an out-group. This analysis involved 37 nucleotide sequences. The numbers on the branches are percent bootstrap support values from 1000 replicates. The scale shows the number of nucleotide substitutions per site between DNA sequences. Representative sequences identified in this study are marked with a dot.



The phylogenetic analysis including only various cervid species revealed that the representative sequences obtained in this study showed high similarity to reference sequences of *Blastocystis* and were assigned to ST10, ST13, and ST14 (Table 2). ST10 clustered with sequences derived from elk, red deer, sika deer, and white-lipped deer. ST13 clustered with sequences from muntjac, reindeer, and forest musk deer, whereas ST14 grouped with sequences isolated from sika deer and elk (Fig. 2). The subtypes identified in this study (ST10, ST13, and ST14), as well as the geographical distribution and zoonotic potential of *Blastocystis* in European cervids, are summarized in Table 3.

Contributing factors

Statistical analysis showed that the best-fitting model included five variables: presence on a wind farm, percentage of arable land, percentage of settlements, percentage of surface water cover, and the study area (Table 4). Roe

Table 2 Comparison of the obtained sequences with GenBank sequences including cervid species.

Representative sequence ID/Isolate	Closest match ID	Host	Origin	Percentage match	Query cover
ST10 PV982219/IL022	MK930356	Sika deer	China	100%	100%
	MK930352	White-lipped deer	China	100%	100%
ST10 PV982234/IL013	MK930356	Sika deer	China	100%	100%
	MK930352	White-lipped deer	China	100%	100%
ST10 PV982241/RA019	MK930356	Sika deer	China	99.81%	100%
	MK930357	Sika deer	China	99.81%	100%
ST13 PV982220/IL023	MF186700	Muntjac	United Kingdom	100%	100%
	MH325366	Reindeer	China	99.14%	100%
ST13 PV982228/WE020	MF186700	Muntjac	United Kingdom	100%	100%
	MH325366	Reindeer	China	99.14%	100%
ST13 PV982244/RA014	MF186700	Muntjac	United Kingdom	100%	100%
	MH325366	Reindeer	China	99.07%	100%
ST14 PV982221/IL025	MK357783	Sika deer	China	98.95%	100%
	MF186664	Elk	United Kingdom	98.95%	100%
ST14 PV982222/RA018	MK357783	Sika deer	China	99.63%	100%
	MF186664	Elk	China	100%	100%
ST14 PV982223/WE003	MK357783	Sika deer	China	98.78%	100%
	MF186664	Elk	China	98.78%	100%
ST14 PV982225/WE006	MK357783	Sika deer	China	98.78%	100%
	MF186664	Elk	China	98.78%	100%

deer on wind farms had a significantly lower probability of *Blastocystis* occurrence than those outside the area (Wald $\chi^2=5.759$, $p=0.016$). Moreover, the probability of *Blastocystis* occurrence in roe deer decreased with increasing percentage of settlements (Wald $\chi^2=4.460$, $p=0.035$) but increased with increasing percentage of arable land and water in the area occupied by them (Wald $\chi^2=6.295$, $p=0.012$ and Wald $\chi^2=5.080$, $p=0.024$ respectively). Other variables, sex and age of animals, and season did not impact the *Blastocystis* occurrence in roe deer and were excluded during model selection.

DISCUSSION

Blastocystis is a common protist of humans and is also frequently observed in wild and domestic animals¹. However, the role of wildlife in *Blastocystis* transmission remains poorly understood, particularly in Poland. Roe deer are widely distributed and frequently inhabit areas overlapping with human, domestic, and wild animal populations. Consequently, they may act as pathogen reservoirs across these interfaces. The clinical pathogenicity of *Blastocystis* remains debated, as colonization is often asymptomatic in both humans and animals^{4,42}.

Although *Blastocystis* is a prevalent enteric protist in wild and captive cervid populations worldwide, our literature review revealed no direct link between specific subtypes and the occurrence of clinical symptoms. High colonization rates have been documented in species such as white-tailed deer or sika deer without any observable veterinary symptoms^{34,35}.

Nonetheless, interest in this protist has grown due to its zoonotic potential and broad genetic diversity⁴¹. Transmission between animals and humans appears to be bidirectional. This route is especially relevant in settings involving close contact, such as among zoo workers, laboratory personnel, and individuals exposed to livestock or shared water sources^{11,41,43}. This transmission link is supported by the identification of identical or closely related subtypes in both hosts^{13,15}. Furthermore, experimental studies have confirmed human-to-animal transmission⁴⁴.

The protist's low host specificity and wide subtype diversity highlight the significance of cross-species transmission among livestock, wildlife, domestic animals, and humans sharing the same environment¹.

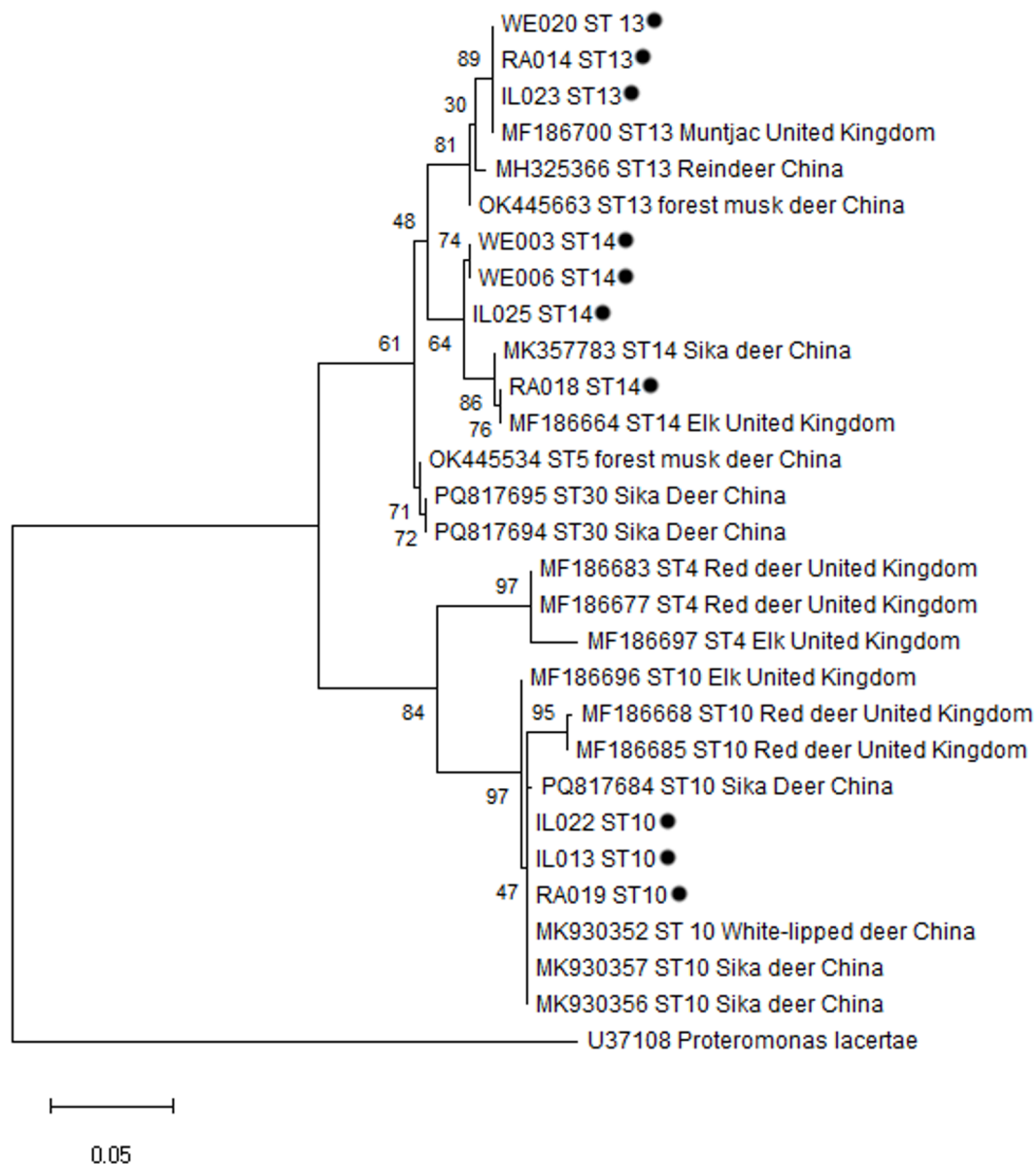


Fig. 2 Phylogenetic tree based on sequences of the partial small subunit ribosomal RNA (SSU rRNA) of *Blastocystis*. The phylogenetic tree was constructed using the maximum likelihood method and K2+G substitution model. Sequence of *Proteromonas lacertae* served as an outgroup. This analysis involved 29 nucleotide sequences. The numbers on the branches are percent bootstrap support values from 1000 replicates. The scale shows the number of nucleotide substitutions per site between DNA sequences. Representative sequences identified in this study are marked with a dot.

To our knowledge, this study represents the largest survey of *Blastocystis* in free-ranging roe deer conducted in Europe to date ($n=151$). The prevalence of 32.5% is comparable to the colonization rate previously documented in wild roe deer populations in Portugal (32.5%) while being noticeably higher than that observed in Spain (12.9%)⁸. The remaining reports from Europe (Denmark, Portugal, United Kingdom) involved only a few individuals^{39,41,45}. Furthermore, the presence of *Blastocystis* in Europe has already been confirmed in several other cervid species, including elk, reindeer, Reeves' muntjac, red deer, and fallow deer^{8,19,42,45–47}.

The isolates obtained in our study align with this trend, indicating close phylogenetic relationships with various cervid species. Within the identified ST13, ST10, and ST14 subtypes, sequences from Polish roe deer clustered

Table 3 Occurrence and zoonotic potential of *Blastocystis* subtypes in Cervidae in Europe.

Host Latin name	Host common name	Prevalence % (no. positive / total)	Blastocystis subtypes	Country	References
<i>Capreolus capreolus</i>	Roe deer	32.5 (49/151)	ST10^c , ST13, ST14^c	Poland	This study
		100 (1/1)	ST10^c	Denmark	Stensvold et al., 2009
		32.5 (13/40)	ST5^b , ST10^c , ST13, ST44, ST49	Portugal	Figueiredo et al., 2025b
		100 (1/1)	ST10^c	Portugal	Santin et al., 2023
		12.9 (12/93)	ST2^a , ST10^c , ST13, ST24, ST49	Spain	Figueiredo et al., 2025b
<i>Alces alces</i>	Elk	50 (1/2)	ST5^b	United Kingdom	Alfellani et al., 2013b
		40 (2/5)	ST4^a , ST10^c , ST14^c	United Kingdom	Betts et al., 2018; Betts et al., 2020
		100 (1/1)	ST10^c	United Kingdom	Betts et al., 2020
		100 (2/2)	ST13, ST14^c	United Kingdom	Betts et al., 2018; Betts et al., 2020
		50 (2/4)	ST4^a , ST10^c	United Kingdom	Betts et al., 2018; Betts et al., 2020
<i>Muntiacus reevesi</i>	Reeves's muntjac	40.4 (38/94)	ST2^a , ST5^b , ST10^c , ST21, ST24, ST25	Portugal	Figueiredo et al., 2025b
<i>Cervus elaphus</i>	Red deer	11.9 (39/329)	ST5^b , ST10^c , ST13, ST14^c , ST21, ST23^c , ST24, ST43, ST44	Spain	Figueiredo et al., 2025b
		100 (1/1)	ST25, ST42	Spain	Santin et al., 2023
		25 (2/8)	ST1^a	Poland	Kaczmarek et al., 2021
		100 (1/1)	ST5^b	Italy	Gabrielli et al., 2021
		5.2 (4/96)	ST5^b , ST10^c , ST14^c , ST21, ST24, ST25, ST26, ST30, ST42, ST43, ST44	Spain	Figueiredo et al., 2025b
<i>Dama dama</i>	Fallow deer				

Potentially zoonotic subtypes are in bold. ^aFrequent subtype shared between humans and animals; ^bModerate common in human; ^cRare common in human.

Table 4 Effects of wind farm (WF), arable land cover (ARABLE), settlement cover (SETTLEMENTS), surface water cover (WATER), and study area (AREA) on the probability of *Blastocystis* presence in roe deer in a generalized linear binary model; other variables (sex, age of animals, and season) were excluded during model selection.

Parameter	Estimate	Standard error	Lower CI	Upper CI	Wald Chi ²	P-value
Intercept	−15.247	5.937	−26.883	−3.610	6.595	0.010
WF (yes)	−1.300	0.542	−2.362	−0.238	5.759	0.016
WF (no)	0*					
ARABLE	0.305	0.122	0.067	0.543	6.295	0.012
SETTLEMENTS	−1.100	0.521	−2.122	−0.079	4.460	0.035
WATER	1.970	0.874	0.257	3.683	5.080	0.024
AREA (Hža)	3.374	1.861	−0.273	7.020	3.288	0.070
AREA (Rawa M.)	−1.001	0.541	−2.069	0.051	3.479	0.062
AREA (Węgrów)	0*					

*reference category

with isolates from wild ruminants in other parts of the world, including British muntjac, red deer, and elk, as well as Chinese reindeer and sika deer.

In addition, phylogenetic analysis confirmed that the isolates belonging to subtype ST14 showed similarity to sequences obtained from domestic ruminants (goats, sheep, and cattle) from Asia, while one of the isolates clustered with a human sequence from Senegal. Similarly, the ST10 isolates showed similarity to sequences from domestic ruminants (goats, sheep, and cattle) and cervids from China, as well as human isolates recorded within

the clade from Senegal. In contrast, the ST13 isolates formed a highly stable, monophyletic clade closely related (99.14–100% similarity) to wild ungulates, including Tibetan antelope, goral, reindeer, and muntjac.

Forty-nine *Blastocystis* subtypes have been reported in the literature^{5–8}. Of those, sixteen subtypes (ST1–ST10, ST12, ST14, ST16, ST23, ST35, and ST41) have been described in humans^{6,36,48}. To date, about seventeen STs, including ST1, ST2, ST4, ST5, ST10, ST13, ST14, ST21, ST23, ST24, ST25, ST26, ST30, ST42, ST43, ST44, and ST49, have been identified in European Cervidae. Eight *Blastocystis* STs, including ST2, ST5, ST10, ST13, ST14, ST24, ST44, and ST49, were identified in *Blastocystis*-positive samples from European wild roe deer (Table 3).

The most prevalent subtype in our study was ST14 (26/49; 53.1%), followed by ST13 (16/49; 32.7%) and ST10 (5/49; 10.2%). In comparison, Figueiredo et al. (2025) identified ST13 as the predominant subtype among roe deer in Portugal (58.8%; 10/17 positive samples) and Spain (43.8%; 7/16 positive samples), while the frequency of ST10 was 5.9% (1/17 positive samples) and 18.8% (3/16 positive samples), respectively, and subtype ST14 was not detected⁸.

Contrary to earlier beliefs, *Blastocystis* subtypes do not appear to be host-specific. In humans, ST1–ST9 and ST12 are commonly reported, with occasional cases of ST10, ST14, and ST16; whereas ST13 has not yet been detected in humans^{37,48,49}.

According to the subtypes detected in this study, subtype 10 has been previously found in human volunteers in Thailand³⁶ and both subtypes 10 and 14 were found in Senegalese children³⁷. Both are typical cattle subtypes^{50–52}. The study by Jinatham et al. (2021) suggests possible zoonotic transmission of some subtypes, including ST10³⁶, but the lack of sharing between animals and their owners indicates that environmental sources such as water or soil are the more likely origin of *Blastocystis* in the examined community³⁶. Jiménez et al. (2022) identified multiple shared subtypes across diverse hosts, as well as individual hosts harboring several subtypes⁵³. For instance, cattle were associated with four distinct subtypes, including ST14. These findings suggest that direct interactions among animals, humans, and their shared environment represent a key transmission pathway for *Blastocystis*⁵³. Numerous studies identify ST10 and ST14 as the predominant subtypes in both wild and domestic ruminants^{10,17,54–56}. Moreover, frequent detection of ST10, followed by ST14, in bovids, cervids, camelids, ovids, and equids points toward host adaptation within ungulate populations¹.

Additionally, Rudzińska et al. (2021) underline that ST13 might exhibit a wide host range, having been identified in primates, marsupials, and artiodactyls. Originally ST13 was recognized in Australia in the quokka (Marsupial)¹⁵, followed by the Western grey kangaroo⁵⁷, the Java mouse-deer⁵⁰, as well as in reindeer in China⁵⁸. Furthermore, in the Polish Gdańsk Zoo, this subtype was detected in all tested lutungs and one guereza¹⁷.

Various transmission routes, human-to-human, zoonotic, and waterborne, have been investigated^{13,39,40,59}, and the presence of certain subtypes in both humans and animals suggests their zoonotic potential^{12,41}. However, defining subtype-specific transmission dynamics requires further investigation, particularly through systematic environmental sampling.

Furthermore, this study investigated the potential effects of anthropogenic pressure (distance to the nearest wind turbine, built-up areas) and landscape structure (arable land, surface water cover) on *Blastocystis* colonization levels in roe deer.

The probability of *Blastocystis* occurrence in roe deer increased with increasing percentage of arable land (Wald $\chi^2 = 6.295$, $p = 0.012$). This relationship is probably associated with exposure of wildlife to fecal-borne pathogens associated with agricultural management. Roe deer frequently forage in cultivated fields, utilizing agroecosystems as a rich food resource. However, such increasingly synanthropic behavior elevates the risk of contact with soil amended with manure or slurry. As indicated by Akdur-Öztürk et al. (2025) and Kuijper et al. (2014)^{60,61}, agricultural areas are characterized by a higher level of environmental contamination with infective stages of parasites. Insufficiently composted natural fertilizers, consisting of livestock excreta and litter, serve as key reservoirs and sources of microbial contamination⁶². Moreover, the application of liquid slurry promotes the long-term persistence of *Blastocystis* cysts in the topsoil, from where they can be easily ingested by foraging roe deer. Cultivated fields promote roe deer aggregation during foraging, leading to higher fecal contamination of

the environment and consequently increasing the risk of protist transmission⁶². Sharing habitats, such as pastures and cropland, with domestic ruminants (cattle, sheep) can lead to the transmission of pathogens from livestock to wildlife¹⁹. Some studies point to the possibility that soil in intensively managed agricultural areas could act as an additional pathway of transmission³⁶.

Moreover, our study shows that the probability of *Blastocystis* occurrence in roe deer was positively associated with the proportion of water bodies in the area occupied by them (Wald $\chi^2=5.080$, $p=0.024$). *Blastocystis* can occur in aquatic environments, suggesting that surface waters serve as both a reservoir and a vehicle for transmission, particularly where wild and domestic animals coexist. Waterborne transmission of *Blastocystis* has long been recognized^{13,59}; for instance, it was detected in untreated drinking water in Peninsular Malaysia⁵⁹, as well as in the water supply of a rural community in central Thailand and the schoolchildren consuming it⁶⁴. Furthermore, *Blastocystis* has been identified in rivers supplying rural communities^{13,63}. In Malaysia, it was detected not only in river water but also in other local sources, including tap, stored, and well water⁶³. Similarly, in Nepal, identical subtypes were recovered from river water, humans, and animals within the same village, providing further evidence of waterborne transmission^{13,65}.

In Europe, the occurrence of *Blastocystis* in water has been associated with symptomatic infection in Italy, where the same subtype (ST1) was detected in both a human stool sample and the implicated water⁶⁶. Water contamination has also been reported elsewhere. Spanish irrigation water contained *Blastocystis* cysts of ST2 and ST4⁶⁷. Similarly, Polish surface waters contained ST1 in lagoons, whereas ST3 was identified in rivers and lakes. Wastewater also poses a transmission risk. Swedish treatment plants revealed *Blastocystis* ST1–ST4, ST8, and ST10⁶⁸. In Scotland, viable cysts were recovered from 29% (9/31) of influent and 9% (4/42) of effluent samples⁶⁹. Importantly, *Blastocystis* cysts can survive for 19 days to 1 month in water at 25 °C. At 4 °C, they can persist for up to 2 months⁷⁰.

Roe deer on wind farms had a statistically significantly lower probability of *Blastocystis* occurrence than those outside the area (Wald $\chi^2=5.76$, $p=0.016$; Table 4). No direct studies link *Blastocystis* in wildlife to wind farms. However, habitat changes and fragmentation may indirectly affect transmission by altering animal behavior (avoidance of the area and selection of alternative migration routes), access to water, and contact patterns^{19,60,71}. In one of the studied areas, a lower density of roe deer was found at the wind farm in summer compared to the adjacent area⁷³.

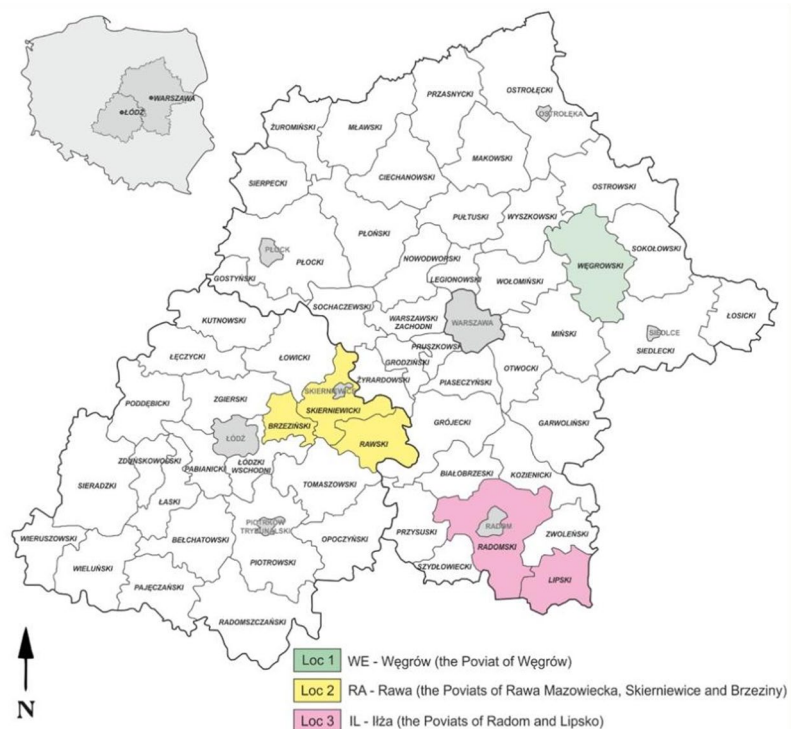
In this study, the probability of *Blastocystis* occurrence in roe deer decreased significantly with an increasing percentage of settlements. (Wald $\chi^2=4.46$, $p=0.035$; Table 4). However, data explaining the relationship between urbanization or settlement density and *Blastocystis* remain limited. The increasing percentage of settlements correlates with higher habitat fragmentation and a significant reduction in the availability of continuous, natural habitats suitable for roe deer. In highly fragmented or suburban landscapes, roe deer populations often exhibit lower local densities or altered spatial distribution to avoid human disturbance and infrastructure^{23,73}.

In conclusion, this study provides the first data on the occurrence and subtype distribution of *Blastocystis* in wild roe deer in Poland. In particular, three *Blastocystis* subtypes were identified. Although two of these subtypes, ST10 and ST14, can be shared with humans, their zoonotic potential remains low. Nevertheless, these findings provide baseline data for monitoring and exploring the transmission dynamics of this protist at the animal–human interface. The increasing interaction between humans and animals, promoted by factors such as agriculture, urbanization, industrialization, and globalization, can influence cross–species transmission and the spread of zoonotic pathogens.

Moreover, our results demonstrate that *Blastocystis* colonization in wild roe deer within the study area may be influenced by landscape structure and anthropogenic factors.

Therefore, further studies investigating the presence of *Blastocystis* in a wider range of domestic and wild animal species from diverse geographic regions are needed. Such studies could improve our understanding of its epidemiology, subtype–host associations, and potential zoonotic transmission patterns.

Fig. 3 Geographic map of Poland, showing three localities of sampling sites.



Materials and methods

Source and sample collection

A total of 151 fecal samples (all consisting of fully formed pellets) from hunter-killed roe deer were collected. The study was conducted in accordance with national guidelines. According to the Animal Protection Law in Poland (Act of the Polish Parliament dated 21 August 1997), the use of samples of animal origin from game animals for scientific research is not a procedure, and the consent of the ethics committee is not required.

The material was obtained in 2023/2024 from three locations: Location 1 (WE–Węgrów, $n=43$), Location 2 (RA–Rawa, Skierniewice, and Brzeziny, $n=45$), and Location 3 (IL–Radom and Lipsko, $n=63$) (Fig. 3). Approximately 10 g of fecal samples were collected from a section of the large intestine during autopsy and placed in a sterile tube with 70% alcohol. The prepared material was stored at -80°C until processing.

Molecular analysis

Prior to molecular analysis, 200 mg of feces from each sample was washed three times with sterile phosphate-buffered saline (PBS) using 2.0 mL microcentrifuge tubes (centrifugation at $2800\times g$ for 5 min). The commercial QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used for genomic DNA isolation. The extracted DNA was dissolved in 40 μL of TE buffer and stored at -20°C for further studies.

Molecular identification of *Blastocystis* was performed by PCR targeting a 607–base pair fragment of the small subunit ribosomal RNA (SSU rRNA) gene, amplified with specific primers RD5 (5′–ATCTGGTTGATCCTG CCAAGT–3′) and BhrDr (3′–GAGCTTTTAACTGCAACAACG–5′). PCR amplification was conducted on a Mastercycler[®] nexus thermal cycler (Eppendorf, Hamburg, Germany) in standard 0.2 mL microtubes according to the following composition of a 25 μL reaction mixture: 12.5 μL of $2\times$ PCR Master Mix Plus (A&A Biotechnology, Gdynia, Poland), 1 μL of each 10 μM primer, 2 μL of genomic DNA and a nuclease-free water. The PCR

reaction conditions were as follows: initial denaturation at 94 °C for 1 min, and then 30 cycles of denaturation, annealing and extension for 1 min at 94 °C, 59 °C and 72 °C, respectively, and a final extension step of 2 min at 72 °C. The PCR products were electrophoresed on 1.5% agarose gels stained with Midori Green Advance dye (Nippon Genetics, Japan) and then visualized using a Molecular Imager® Gel Doc™ XR System (Bio–Rad, Hercules, California, USA).

The positive PCR products were purified with Clean Up (A&A Biotechnology, Gdynia, Poland) and then subjected to bidirectional sequencing by the Macrogen Humanizing Genomics Europe (Amsterdam, The Netherlands). The obtained nucleotide sequences were assembled using BioEdit Sequence Alignment Editor v. 7.2 and subsequently analyzed by the BLAST–NCBI program (<https://blast.ncbi.nlm.nih.gov/Blast>).

Phylogenetic analyses

The phylogenetic analyses for *Blastocystis* were based on the SSU rRNA (small subunit ribosomal RNA). The trees were constructed using 10 representative sequences. Three sequences belong to ST10 and ST13 and 4 sequences belong to ST14. A *Proteromonas lacertae* sequence was used as an outgroup to root the trees. Sequences were aligned using the ClustalW algorithm in Mega X and trimmed at the ends using BioEdit v. 7.2⁷⁴. The trees were constructed using Mega X software⁷⁴. The maximum likelihood (ML) method was applied using the Tamura 3–parameter (T92+G). The analysis involved 37 and 29 nucleotide sequences, respectively. Accordingly, there were 190 and 460 positions in the final dataset. In both analyses, the internal node support was assessed by 1,000 bootstrap sampling. The scale bar indicates the equivalence of distance between sequences.

Data elaboration and statistics

Based on the hunters' reports, basic information was determined for each sample: age of roe deer, sex of roe deer, season of sample collection, and study area (Iłża, Węgrów, Rawa Mazowiecka). Based on the roe deer location, their presence in the wind farm area was determined. The wind farm area was defined as the interior of the area surrounded by a compact group of wind turbines and within a buffer of 700 m from the outermost wind turbine, in accordance with previously assumed distances indicating the impact of this infrastructure on roe deer^{75–77}. Next, for each hunting district where roe deer were hunted, the percentage of selected land cover types was calculated. Land cover structure was calculated based on the publicly available Corine Land Cover (CLC) database. For the study, four main land cover types were selected: (1) arable land, (2) forests, (3) water, and (4) settlements. The percentage of each cover type was calculated in QGIS 3.22 by overlaying the hunting district boundaries and the acquired CLC layers. The correlation of the percentage of calculated cover types was then assessed to eliminate collinearity between explanatory variables in the model. A standard threshold correlation coefficient of $|r| = 0.7$ was adopted. Forests showed a high correlation with arable land; therefore, forests were excluded from further analysis.

A statistical analysis of the *Blastocystis* occurrence in roe deer was performed using a generalized linear binary model in SPSS software. The dependent variable in the model was the presence or absence of *Blastocystis* in roe deer, expressed as a binary variable. The probability of the *Blastocystis* occurrence in roe deer was assessed based on eight variables including animal characteristics, season, sampling location, and environmental conditions: (a) roe deer sex, (b) roe deer age, (c) season, (d) study area, (e) presence of roe deer in the wind farm area, (f) percentage of arable land, (g) percentage of water, (h) percentage of settlements. We performed a full model selection procedure for all model variants (with various variable numbers and compositions), including the null, intercept–only model, to determine the model that best fits the data. We assessed the quality of the models using the Akaike Information Criterion (AIC), where lower values indicate a better model fit. Ultimately, we presented the best–fitting model, i.e., with the set of variables that resulted from the analysis of the AIC values.

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Author contributions JK and ML: wrote the main manuscript text; JK: prepared Figs. 1, 2 and 3; Tables 1 and 3, molecular analysis; ML: prepared Table 2; DK: prepared Table 4, statistical analysis; MK and MS: collecting the samples. All authors reviewed the manuscript.

Data availability The nucleotide sequences generated in the present study have been deposited in GenBank (<https://www.ncbi.nlm.nih.gov/>) under accession numbers PV982219–PV982267. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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